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Syngas biomethanation by co-digestion with brewery spent yeast in a lab-scale reactor

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ABSTRACT

The achievement of the current decarbonization goals requires optimizing biomass conversion processes to reduce processing costs and unlock the valorization of a wide array of feedstocks. Gasification enables exploiting recalcitrant biomasses to produce syngas which can be upgraded to biomethane. In this context, the possible integration of syngas biomethanation and waste treatment into a single unit is particularly relevant. This study investigates the methanation performance of the co-digestion of syngas (45% H_2, 20% CO, 25% CO_2, 10% N_2) and brewery spent yeast (BSY) in a lab-scale continuously stirred tank reactor (CSTR) operated for 85 days, with the aim of evaluating the microbial culture capacity to simultaneously convert syngas and BSY and the stability of the process over different stages. Adaptation of mixed microbial cultures was performed both in batch bottles and by continuous feeding of syngas in a CSTR reaching after 44 days a methane productivity of 150 $mL \cdot L^{-1} \cdot d^{-1}$ and a 27% degradation of the initial COD. The adapted culture in CSTR was then subjected to a continuous syngas load of 1.13 $L \cdot L^{-1} \cdot d^{-1}$ and 0.55 $g_{VS} \cdot L^{-1} \cdot d^{-1}$ of BSY, reaching methane productivity of up to 233 $mL_{CH4} \cdot L^{-1} \cdot d^{-1}$. The H_2 and CO conversion efficiencies were on average over 80% towards the end of the adaptation stage and during the 21 days of the continuous liquid feeding stage. While sulfur limitation inhibited methanogenic activity after 21 days of co-feeding of syngas and BSY, satisfactory process stability was achieved and efficient syngas conversion by microbial cultures could be demonstrated in concomitance with a considerable organic load reduction during the previous stages. The study demonstrates that BSY is a valuable substrate for syngas co-digestion, with a high potential for process scale-up under optimized operating conditions. Future work should optimize the methane productivity especially by focusing on secondary nutrients management.

1. Introduction

The transition towards a decarbonized energy system requires a concomitance between electrical energy conversion technologies and biofuels with high volumetric energy densities. While the deployment of electrical infrastructure powered by renewable electricity depends on the development of large-scale renewable generation infrastructure, the production of drop-in biofuels in relevant quantities relies on optimizing the valorization of a variety of carbonaceous feedstocks. Such optimization in turn requires the intensification of engineered systems capable of converting low-cost residual biomass to biofuels. Contemporary biomass-to-biofuel technologies encompass both processes applicable to easily biodegradable biomass, such as biological technologies, and processes applicable to hardly degradable substrates, such as thermochemical technologies. Significant expansions of the global biofuel production capacity will rely on the simultaneous development of both biological and thermochemical conversion technologies capable of addressing resource diversity. A fundamental product of interest in this context is biomethane, a drop-in gaseous biofuel with a high volumetric energy density that can be used in substitution to fossil natural gas. Particularly in the European context, biomethane is considered a critical biofuel for the energy transition, as witnessed by the 35 billion cubic

Abbreviations: BSY, Brewery Spent Yeast; COD, Chemical Oxygen Demand; CSTR, Continuously Stirred Tank Reactor; C. R., Control Reactor; D., Day; HRT, Hydraulic Retention Time; L_b, Volume (Liter) of reactor bed; L_r, Volume (Liter) of reactor; MFC, Mass Flow Controller; OLR, Organic Loading Rate; PCA, Principal Component Analysis; OTU, Operational Taxonomic Unit; VS, Volatile Solids; TS, Total Solids; T. R., Test Reactor.

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meters annual production target recently set out by European authorities [1].

On the one hand, a global expansion in biomethane production capacity can be achieved through enhanced exploitation of easily degradable matrices in anaerobic digestion processes, that allows the conversion of carbonaceous waste streams to biomethane and carbon dioxide. Such expansion should be supplemented by innovative process solutions that can increase biomethane yields and expand the spectrum of treatable substrates through appropriate pre-treatment methods. On the other hand, thermochemical technologies capable of converting hardly biodegradable carbonaceous matrices to combustible syngas have a fundamental role to play in this space. As a specific example, the coupling of thermochemical biomass gasification with downstream conversion to biofuels has the potential to unlock substantial quantities of renewable carbon as a feedstock for drop-in biofuels that can progressively displace fossil fuels employed in hard-to-electrify applications.

Such opportunity can be especially relevant when total process costs can be minimized by coupling cost-intensive waste treatment processes with biofuel production processes, as in the case of organic waste treatment with biomethane recovery through anaerobic digestion, which already benefits from full commercial maturity. In this context, the further optimization of biofuel production coupled with waste treatment represents a major interest in research.

One key application of such concept is the integration between syngas conversion to biomethane and anaerobic waste treatment in a single bioreactor. The process relies on the high-temperature ex-situ conversion of woody biomass to syngas, and the subsequent microbial conversion of syngas to biomethane. Such integration could deliver the twofold benefit of increasing biomethane production per unit of reactor volume through the addition of a supplemental biologically convertible carbon stream (syngas) while reducing total process costs per unit of waste treated. The integration between gasification and methanation of syngas has been widely investigated in the literature, especially in relation to the use of catalytic methanation as the main syngas conversion technology [2,3]. In recent years, though, increasing attention has been dedicated to the biological methanation of syngas thanks to its robustness and low severity characteristics. These include its high tolerance to feed gas impurities and its mild operating temperatures and pressures, which can be set as low as 35 °C and atmospheric pressure, respectively [4].

Syngas biomethanation consists of a set of biochemical reactions including direct and acetate-mediated methanation of H_2, CO, and CO_2, yielding a maximum molar conversion of 0.25 mol of CH_4 per mol of CO and H_2, independently from the reaction pathway [5]. Recent research has focused on the performance of syngas biomethanation in a variety of setups, demonstrating promising syngas conversion rates. More specifically, Asimakopoulos et al. [6] operated a lab-scale trickle-bed reactor producing 2.1 $L_{CH4}{\cdot}L_b^{-1}{\cdot}d^{-1}$ and 4.98 $L_{CH4}{\cdot}L_b^{-1}{\cdot}d^{-1}$, at mesophilic (37 °C) and thermophilic (60 °C) conditions, respectively, under a gas loading rate of 40.00 $L{\cdot}L_b^{-1}{\cdot}d^{-1}$. On the other hand, syngas biomethantion in lab-scale continuously stirred tank reactors (CSTR) has been also investigated at different operating conditions yielding methane productivities in the range 1.26–3.96 $L_{CH4}{\cdot}L_r^{-1}{\cdot}d^{-1}$ under syngas loadings in the range 5.76–18 $L{\cdot}L_r^{-1}{\cdot}d^{-1}$ [7–9]. At the pilot-scale, Asimakopoulos et al. [10] coupled a trickle bed bioreactor with a fluidized bed steam gasifier, demonstrating productivity of up to 10 $L_{CH4}{\cdot}L_b^{-1}{\cdot}d^{-1}$ methane and a 98% conversion of CO and 100% of H_2 at a 72 $L{\cdot}L_b^{-1}{\cdot}d^{-1}$ syngas inflow rate, whereas Sun et al. [11] obtained a 0.42 $L_{CH4}{\cdot}L_r^{-1}{\cdot}d^{-1}$ methane productivity in a bubble column reactor fed with 0.2 $L{\cdot}L_r^{-1}{\cdot}d^{-1}$ pyrolysis syngas and additionally glucose. Recent studies have also assessed the capacity of mixed microbial cultures to convert CO-containing syngas alongside soluble waste substrates: Luo et al. [12] operated a tank reactor for co-digestion of CO and sewage sludge achieving simultaneously full CO conversion and 40% of solids reduction showing no inhibition on sludge digestion with a CO pressure up to 1.58 atm. Furthermore, Yang et al. [13] co-digested food waste and a gas mixture with an H_2:CO ratio of 5:4, obtaining up to 1.75 $L_{CH4}{\cdot}L_r^{-1}{\cdot}d^{-1}$ under a continuous gas loading rate of 1.33 $L{\cdot}L_r^{-1}{\cdot}d^{-1}$ and an organic loading rate of 3.52 $g_{VS}{\cdot}L_r^{-1}{\cdot}d^{-1}$ in a lab-scale CSTR. Analogous results were also obtained by Andreides et al. [14] in the continuous co-digestion in lab-scale CSTR of anaerobic sludge and H_2 – CO mixture. These studies show relatively high conversion rates in standalone syngas biomethanation processes and in co-digestion processes demonstrating the successful integration between syngas biomethanation and COD conversion of mixed soluble substrates derived from waste streams. These experimental results, though, need to be interpreted considering the global techno-economic feasibility of biomethane production from possible scale-ups of these processes.

The techno-economic performance of syngas biomethanation integrated into different process configurations (based on Asimakopoulos et al. [10]) was studied by Menin et al. [15], showing that the lowest expected biomethane minimum selling price is 1.63 $€{\cdot}m^{-3}$ and concluding that, under the current state of technology, intensive subsidization would be required to make syngas biomethanation competitive in the energy market. Such findings highlighted that optimization of biomethane production processes is urgently needed to achieve European and global decarbonization goals cost-effectively. Integrated waste-syngas co-digestion systems suitable for biomethane production represent a possible optimization strategy in this context, as partially demonstrated by the existing literature. However, further experimental investigation into such integrated concepts is necessary. In particular, there is a need to evaluate the performance of such integrated systems in relation to specific waste substrates of interest and to assess the potential of the co-digestion of waste and biomass-derived syngas as an efficient biomethane production technology. Such assessment should evaluate the practicality of the full process value chain, from microbial cultures adaptation to continuous waste and syngas treatment, as relevant to real industrial contexts.

This study focuses specifically on the use of brewery spent yeast (BSY) as a waste stream of interest for use in low-cost co-digestion processes, due to its high generation rates (i.e., 1–3% of the produced beer volume worldwide), its high biogas production potential, and nutrients richness [16–18]. Despite its potential for high low-cost methane production from diversified biomass sources, the integrated digestion of BSY and syngas in a single reactor unit remains a challenging research topic, particularly due to the complexity of BSY as substrate and nutrients source [17,18]. Within this space, there is a specific need to define and assess methodologies for both the adaptation of mixed microbial consortia and the continuous co-digestion of the two substrates, aiming at the definition of adequate process conditions for future scale-ups. These gaps were addressed in the present study by first testing the ability of mixed microbial cultures to metabolize CO-rich syngas in the presence of BSY in batch bottle experiments. Secondly, a continuous microbial adaptation method was tested in a lab-scale CSTR with continuous syngas feeding. Thirdly, the cultures adapted under continuous gas supply were also subjected to the continuous feeding of liquid to test the ability and the stability of the system to convert both syngas and BSY under continuous conditions.

2. Materials and methods

The study consisted of two separate experimental campaigns. The first part, as a preliminary study, aimed at assessing the adaptation of the culture to syngas in the presence of BSY in sealed anaerobic bottles by measuring both methane productivity and COD degradation. On the other hand, the second part aimed at culture adaptation to the digestion of a continuous syngas load in the presence of BSY directly in an anaerobic CSTR, whereas the process stability in the reactor was assessed under simultaneous continuous feeding of syngas and BSY. Methane productivity and syngas conversion were measured over all the reactor operational stages while the culture microbial composition was

also analyzed on selected samples to further prove its capability to co-digest syngas and BSY.

2.1. Inoculum and substates

The anaerobic microbial inocula for the experiments were collected from digestate produced at a food waste anaerobic digestion plant located in Lana (South Tyrol, Italy) operated at mesophilic conditions. Brewery yeast collected in a brewing facility located in Lagundo (South Tyrol, Italy) was used as the BSY source. After collection, brewery yeast was autoclaved at 120 °C for 20 min and stored in a dark environment at 4 °C to inhibit biological activity during storage and sample preparation. BSY was characterized in terms of total COD, total N, NH_4-N, total P, VS, and TS (Table 2). The gaseous substrate was artificial syngas with volumetric composition of 25% CO_2, 20% CO, 45% H_2, 10% N_2 (Air-liquide) as a representative mixture of syngas produced by oxy-steam gasification of biomass [19].

2.2. Experimental setup

2.2.1. Preliminary study of microbial culture adaptation

Culture adaptation was performed in three 1.2 L bottles kept in a rotary shaker incubator operated at 150 rpm and 37 °C. The bottles were sealed with pierceable methyl rubber stoppers fixed with screw lids and wrapped with aluminum foil to ensure a lightless environment. The volume of the liquid phase was set at 500 mL while the volume of the gaseous phase was 700 mL. All bottles were inoculated with 400 mL digestate. One bottle (C1) contained the control culture, i.e., BSY was the sole substrate; the remaining two bottles B1 and B2 were fed at identical conditions with BSY and syngas as substrates. BSY was dosed in the same quantity for C1, B1, and B2 at the beginning of the experiment. The ratio between the VS of BSY and the VS of digestate was set as 0.53, based on a previous study on BSY anaerobic digestion [16]. After flushing with nitrogen, the headspace of the bottles was filled up to 1 atm with N_2 in C1 and with syngas in B1 and B2. Commercial phosphate buffer solution was dosed in each bottle to a concentration of 0.05 mol·L^{-1} to keep neutral pH. During the first 30 days, the gas phase was sampled and replaced every second day, between days 30 and 40 once a day, and between days 40 and 45 twice per day. After this time, the cultures were regarded as adapted to syngas conversion based on the measured methane and syngas consumption rates.

After the 45th day of adaptation, when stable methanogenic activity had been observed for the previous 5 days, the liquid phases of B1 and B2 were merged and placed in a stirred tank reactor (*BioBench* purchased from BIOSTREAM) configured as depicted in Fig. 1, forming 1 L of liquid volume over 2.1 L of free space. The reactor headspace was first flushed with nitrogen for 5 h and subsequently, syngas was fed continuously at 1.16 mL·min^{-1}. The gas was injected in the reactor through a sparger consisting of a stainless-steel tube with 15 holes (diameter of 0.6 mm) placed below the lower stirrer (see Fig. 1). The stirring system consisted of two stainless-steel 6-bladed Rushton impellers placed as depicted in Fig. 1. The impeller speed was maintained at 200 or 300 rpm (according to the stage of the experiment, see Table 1) to ensure adequate mass transfer rates. The reactor temperature was kept at 37 °C and controlled through warm water flowing continuously in the vessel jacket. The pH was monitored with a sensor, and it remained in the range 7.5–7.9 although no adjustment was applied. The reactor operated for 22 days to test syngas conversion and methanogenic activity.

Table 2
Characterization of BSY and of the liquid phase in the Test and Control Reactor at selected days during the adaptation stage of the experiment on continuous syngas co-digestion with BSY in lab-scale reactors.

	BSY	Day 0 Test and Control Reactor	Day 49 Test Reactor (End Stage 1)	Day 49 Control Reactor (End Stage 1)
Total P [g·L^{-1}]	2.28 ± 0.05	0.68 ± 0.02	0.57 ± 0.03	0.51 ± 0.00
Total N [g·L^{-1}]	9 ± 1	4.5 ± 0.4	4.26 ± 0.05	4.10 ± 0.08
NH_4-N [g·L^{-1}]	0.61 ± 0.00	2.4 ± 0.3	2.89 ± 0.10	2.65 ± 0.5
VS [g·L^{-1}]	120.1 ± 0.5	19 ± 1	13.2 ± 0.2	12.5 ± 0.2
TS [g·L^{-1}]	130.1 ± 0.5	29 ± 1	23.4 ± 0.2	23 ± 1
COD [g·L^{-1}]	260 ± 20	34.5 ± 0.9	26.1 ± 0.5	22 ± 4

2.2.2. Continuous syngas co-digestion with BSY in a lab-scale CSTR system

Two identical reactors with the characteristics described in Section 2.2.1. were inoculated with 1.33 L digestate. In both reactors, the liquid phase had a total volume of 1.5 L containing inoculum, BSY, and distilled water. BSY was dosed according to a ratio between volatile solids of BSY and anaerobic sludge equal to 0.53 as in the previous experiment. The Test Reactor was fed with syngas while the Control Reactor was fed with N_2. Both cultures were kept at 37 °C to ensure mesophilic conditions. The pH was monitored manually and adjusted with injections of 1 mol·L^{-1} sulfuric acid and sodium hydroxide solutions to maintain a range between 6.8 and 7.8. The first stage of the experiment consisted of the adaptation of syngas-converting bacteria according to an analogous procedure applied for adaptation in batch bottles. The syngas flow in the Test Reactor was increased stepwise: during the first 7 days of experiment the syngas flow was kept at 0.15 mL·min^{-1}, from day 7–19 at 0.30 mL·min^{-1}, from day 19–26 at 0.60 mL·min^{-1}, from day 26 onwards at 1.18 mL·min^{-1}. 4 mL liquid samples were taken regularly three times per week. No liquid substrate substitution was operated in the two reactors for the duration of the adaptation experiment. The Control Reactor was operated in the same way except for the N_2 gas inflow rate, which was set not exceeding 0.32 mL·min^{-1} to avoid excessive dilution of methane in the outflowing gas.

The second stage of the experiment was initiated on day 49 when syngas conversion and methane reached high and stable values. In this

Table 1
Operating conditions in the Test Reactor and Control Reactor during the experiment on continuous syngas co-digestion with BSY.

		Stage 1				Stage 2
Test Reactor		Stage 1.1 Days 0–7	Stage 1.2 Days 7–19	Stage 1.3 Days 19–26	Stage 1.4 Days 26–49	Days 49–69
	Gas inflow [mL·min^{-1}]	0.15	0.30	0.60	1.18	1.18
	Stirring speed [rpm]	200	200	300	300	300
	HRT [d]	-	-	-	-	14
	OLR [g_{VS}·L_{Liquid}^{-1}·d^{-1}]	-	-	-	-	0.55
Control Reactor	Gas flow [mL·min^{-1}]	0.16	0.32	0.16	0.16	0.16
	Stirring speed [rpm]	200	200	300	300	300
	HRT [d]	-	-	-	-	14
	OLR [g_{VS}·L_{Liquid}^{-1}·d^{-1}]	-	-	-	-	0.55

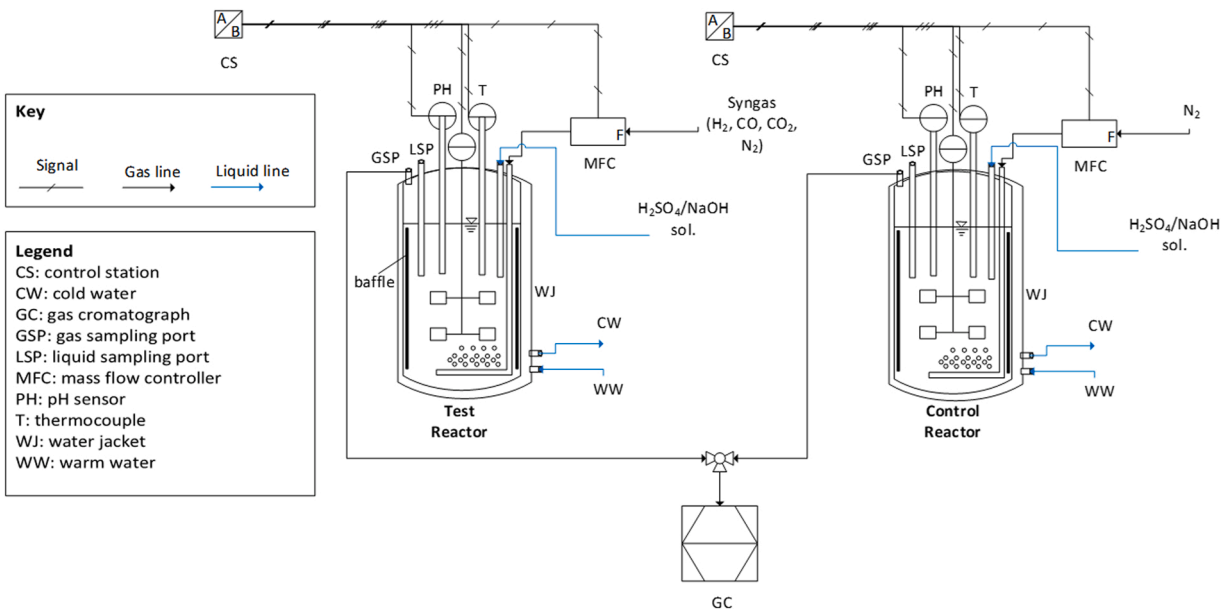

Fig. 1. Schematic representation of the experimental setup used for the experiment on continuous syngas co-digestion with BSY (explained methodology in Section 2.2.2). To test the methanogenic activity of batch-adapted cultures only one reactor was used (explained methodology in Section 2.2.1).

stage, BSY was fed in semi-continuous mode, i.e., by replacing a substrate volume fraction with BSY daily. The BSY was diluted to obtain a feed solution with a concentration of solids equivalent to 7.7 $g_{VS} \cdot L^{-1}$. The applied organic loading rate was 0.55 $g_{VS} \cdot L_{Liquid}^{-1} \cdot d^{-1}$ whereas the hydraulic retention time was 14 days, given a liquid substitution rate of 0.150 $L \cdot d^{-1}$ on five days per week. Other operating parameters were kept constant as in the last step of stage one. The operating conditions applied in the different stages of the experiment are summarized in Table 1.

2.3. Analytical equipment and methodology

The input gas to each reactor was dosed with a MFC (Bronkhost F-201CB). The produced gas volumetric composition was analyzed online by means of a μGC SRA3000 equipped with two columns: a CP-Molesive 5 Å and a PoraPLOT-U. The μGC was pre-calibrated with calibration cylinders (Air Liquide) filled with H_2, CO, CO_2, N_2, and CH_4. The number of moles of the single components was calculated by means of the ideal gas law and taking the input moles of N_2 as reference. The total headspace pressure of the batch bottles was measured using a manometer equipped with a needle. Characterizing parameters of the liquid phase, i.e., total COD, total P, total N, NH_4-N were determined using NANOCOLOR reagent kit, photometer Plus,and thermoreactor. Volatile and total solids were determined according to the standard procedure for water samples [20]. The concentration of volatile fatty acids (acetic acid, propionic acid, butyric acid) in liquid samples was determined with a GC-MS (Gas Cromatography-Mass Spectrometry) system, specifically 7890 A GC SYSTEM 5975 Series MSD AGILENT equipped with a MEGA-WAX Spirit- 0.18 mm-0.3 μm - 40 m- Crossbound column. Each analysis was carried out on 1 μL sample with column heating ramp set at 20 °C·min^{-1} from 40 °C to 90 °C, then 5 °C·min^{-1} to 145 °C, then 20 °C·min^{-1} to 240 °C, then hold for 2 min with a total column residence time of 20.5 min

2.4. DNA isolation and microbial community composition analysis

Microbial composition analysis was performed on samples collected during the experiment on continuous syngas co-digestion with BSY in the CSTR system (Section 2.2.2) at day 0 (inoculation), day 49 (end of adaptation stage), day 67 (after 1.3 HRT of BSY co-feeding) from both the Test and Control Reactor. DNA extraction, library preparation, sequencing, and metataxonomic data analysis were carried out according to Vasmara et al. [21]. DNA extraction was performed using the Powers Soil Pro DNA Isolation Kit (QIAgen) following the manufacturer's recommendations. Gene amplification targeting 16 S rRNA regions V3 and V4 was performed with the bacterial primer set 341 F: 5′-CCTACGGGNGGCWGCAG – 3′ and the 805 R: 5′- GACTACNVGGGTWTCTAATCC – 3′ according to Klindworth et al. [22]. The MICCA v. 1.7.2 pipeline was used for the preprocessing of raw sequencing data and taxonomic assignment was performed with the SilvaDB software version V.132. Alpha diversity (within-sample entropy) was computed using the R package *vegan* version 2.6–4 [23] while principal component analysis (PCA) was carried out on the matrix quantifying the fractions of OTU (operational taxonomic unit) abundance of the identified genera in the analyzed samples. PCA was carried out to compare the culture compositions between samples and was performed using the R function *prcomp*.

3. Results and discussion

3.1. Preliminary study of microbial culture adaptation

3.1.1. Batch culture adaptation

During the 45 days of incubation, the gas phase of cultures C1 (control, BSY as sole substrate), B1, and B2 (BSY and syngas as substrates) was fed at regular intervals with a progressively increasing syngas loading rate as explained in Section 2.2.1. On the other hand, the liquid phase was run in batch mode. The methane production rate, displayed in Fig. 2a, was calculated by linear regression of the cumulative measured methane production during the selected periods. The methane production rate of culture C1 during the last 5 days of incubation was equivalent to 0.3 times the methane production rate observed in the first 7 days, indicating a significant consumption of the liquid substrate (Fig. 2a). On the other hand, the methane production rates, observed for cultures B1 and B2 during the last 5 days of incubation, were 2.4 times higher compared to the initial methane production and 12 times higher than the methane production of culture C1 (Fig. 2a). These observations, along with the fact that the syngas consumption of B1 and B2 increased with increased syngas loading rate, indicated adaptation of the cultures to the methanogenic metabolism of

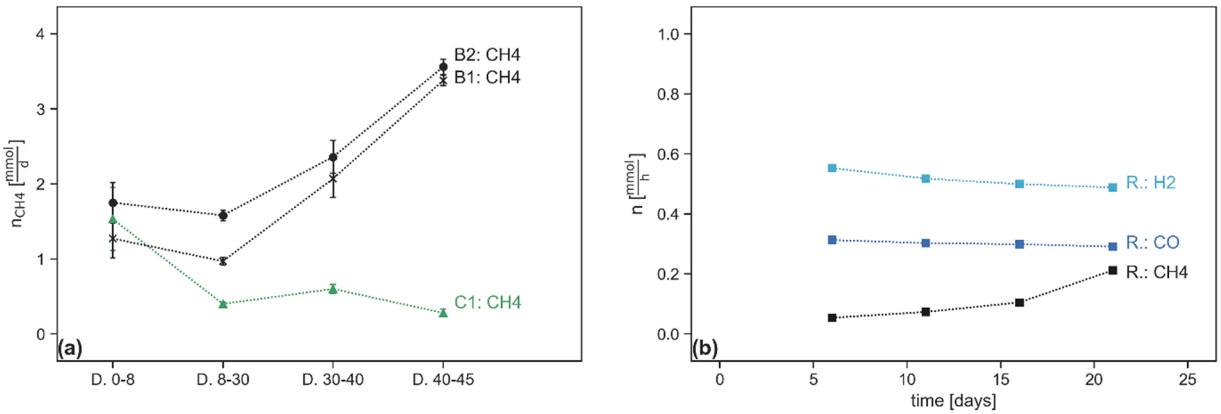

Fig. 2. (a) Methane production rate of culture B1, B2, and C1 during the stages of adaptation in batch mode; (b) methane production rate and outflow rate of unconverted CO and H_2 moles of cultures B1 and B2 in the continuously stirred tank reactor under continuous syngas feeding. Error bars in (a) represent the standard error of regression. Datapoints in (b) correspond to the mean of the measured molar flows in 48 measurements taken over the selected day. Error bars in (b) are hidden by the datapoints and represent the standard error of the mean.

syngas in presence of BSY. Thus, the experiment was pursued by transferring the adapted cultures B1 and B2 in a CSTR to assess syngas conversion to methane under continuous syngas supply.

3.1.2. *Methanogenic performance of adapted cultures*

As described in Section 2.2.1, the cultures B1 and B2 were used as inocula for a CSTR which was operated for 21 days to assess the methanogenic performance of the culture given a constant syngas inflow of 1.16 mL·min^{-1} (3.1 mmol·h^{-1}) while the liquid phase was kept in batch mode. As shown in Fig. 2b, the methane production rate increased over time from 0.05 $mmol_{CH4}·h^{-1}$ up to 0.2 $mmol_{CH4}·h^{-1}$ (equivalent to 114.17 $mL_{CH4}·L^{-1}_{Liquid}·d^{-1}$), while the unconverted output molar flows of H_2 and CO remained relatively constant around 0.5 and 0.3 mmol·h^{-1} respectively (corresponding to H_2 and CO conversion rates of 64% and 52%, respectively). This observation indicates that the adapted cultures could convert the liquid substrate increasingly over time. Indeed, over the 21 days of reactor operation, COD concentration analyses indicated a 23% reduction of the organic load (i.e., from 36.0 ± 2.0 $g_{COD}·L^{-1}_{Liquid}$ to 27.8 ± 1.8 $g_{COD}·L^{-1}_{Liquid}$) while the acetic acid concentration remained constant around a mean value of 24.4 $mmol·L^{-1}_{Liquid}$ excluding the

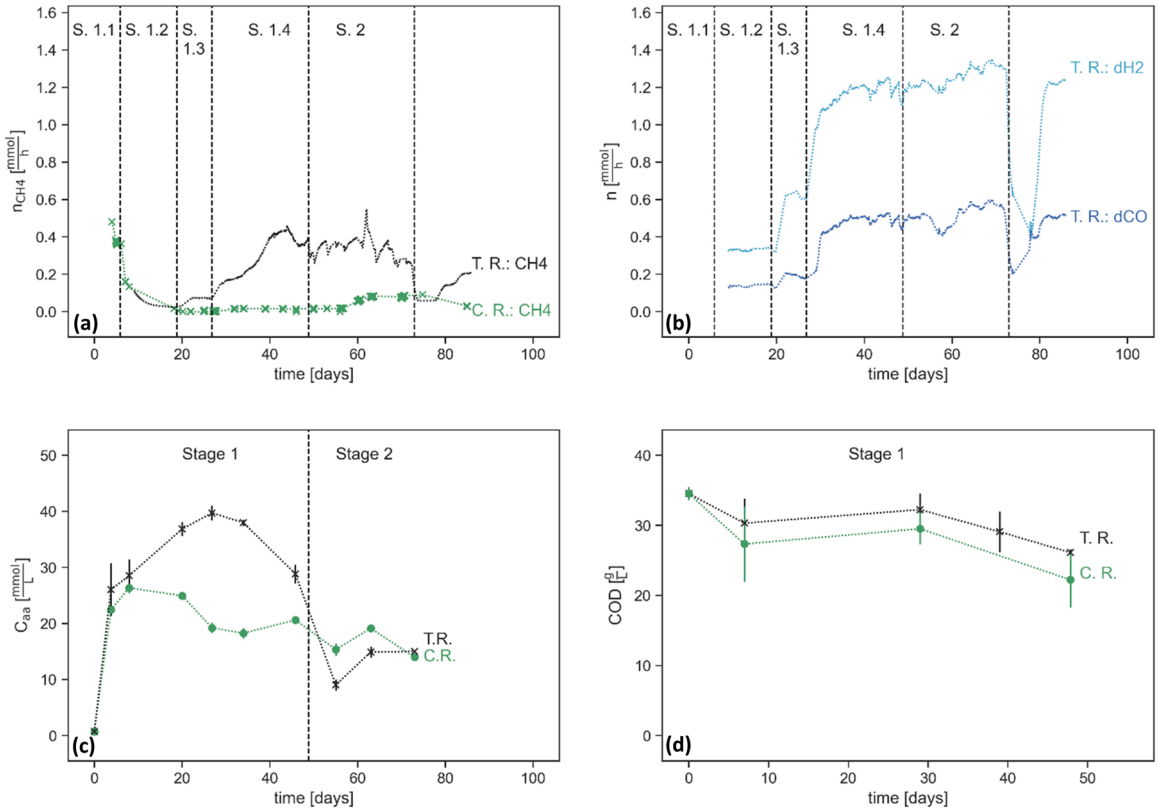

Fig. 3. (a) CH_4 production rate in the Test (T. R.) and Control Reactor (C. R.) during Stage 1, i.e., adaptation by a step-wise increment of syngas at the beginning of each substage (vertical dashed lines), and Stage 2, i.e., continuous BSY co-feeding with syngas; (b) H_2 and CO consumption rate in the Test Reactor at each stage of the experiment; (c) acetic acid concentration over time in the Test and Control Reactor during stage 1 and 2; (d) COD concentration over time in the Test and Control Reactors during stage 1. The operating conditions at each stage of the experiment are outlined in Table 1.

possibility of depletion of accumulated acetate.

This second phase of the preliminary study of culture adaptation thus demonstrated a clear methanogenic activity that confirmed a successful adaptation as well as the ability of the adapted cultures to continuously convert syngas in the presence of BSY. The observed COD conversion also indicated that liquid substrate metabolism was active in the presence of syngas and delivered a considerable reduction of the initial organic load. Such observations allowed taking the experiment to its subsequent stage, i.e., to replicate culture adaptation to syngas digestion in presence of BSY directly in a CSTR and to evaluate the overall stability of a continuous process.

3.2. Continuous syngas co-digestion with BSY in lab-scale reactors

Culture adaptation to syngas biomethanation, as described in Section 2.2.2, was carried on directly in a lab-scale CSTR (Test Reactor) under continuous syngas flow and was then followed by the continuous feeding of BSY. The Control Reactor was operated under the same conditions but using nitrogen instead of syngas.

3.2.1. Culture adaptation

During the first 49 days of operation (Stage 1, Fig. 3a), i.e., during culture adaptation under continuous syngas inflow, the trend of the methane production rate in the Test Reactor generally followed the evolution of syngas inflow, thus increasing over time, except for the first ten days. During this initial period, both reactors showed a progressive decline in methane production rate, likely due to the depletion of easily degradable organic matter, as suggested by the relatively rapid drop in COD (Fig. 3d), from 34.5 to 30.3 $g_{COD}{\cdot}L^{-1}_{Liquid}$ in the Test Reactor and from 34.5 to 27.3 $g_{COD}{\cdot}L^{-1}_{Liquid}$ in the Control Reactor. Culture adaptation to syngas metabolism was then demonstrated by the increasing methane production rate observed between days 10 and 44 in the Test Reactor, that corresponded to an increment in H_2 and CO consumption (Fig. 3b) up to 1.3 and 0.5 $mmol{\cdot}h^{-1}$, respectively. Such increase in gas-phase reducing species consumption demonstrated the primary role of syngas-derived H_2 and CO in methane formation, which is further supported by the sharp decrease in methane production rate that took place during the first 20 days when COD depletion limited methane production. These observations can be also visualized in Fig. 4 in which the significant contribution of H_2 and CO conversion can be recognized by looking at the cumulative methane production in the Test Reactor compared to the methane production from residual COD in the Control reactor between days 30 and 49.

An evaluation of acetic acid accumulation trends showed that the acetic acid concentration in the Test Reactor (Fig. 3c) increased steadily during the first 20 days, peaking between days 20 and 35, and declining subsequently. The converse evolution of methane production between days 20 and 35 indicates that intensive acetogenic activity preceded an aceticlastic methanogenesis phase which allowed methane productivity to grow significantly in the subsequent period (days 35 – 44). This observation is in line with the prevalence of acetogenic metabolism previously observed in the initial phase of syngas exposure in mesophilic syngas biomethantion studies, which demonstrated the use of acetate as a fundamental intermediate to methanogenesis [13,24]. In fact, while both reactors showed a rapid increase in acetic acid concentration shortly after inoculation, the liquid phase in the Test Reactor showed a substantially higher value in acetic acid concentration (up to 40 $mmol{\cdot}L^{-1}$ on day 27) in comparison to the Control Reactor, indicating that syngas carbon and electron moles were the source of half of the acetate moles observed on day 27 in the Test Reactor. On the other hand, over the whole adaptation stage, the butyric and propionic acid concentrations remained stable at low concentrations (around 1 $mmol{\cdot}L^{-1}$) in both reactors.

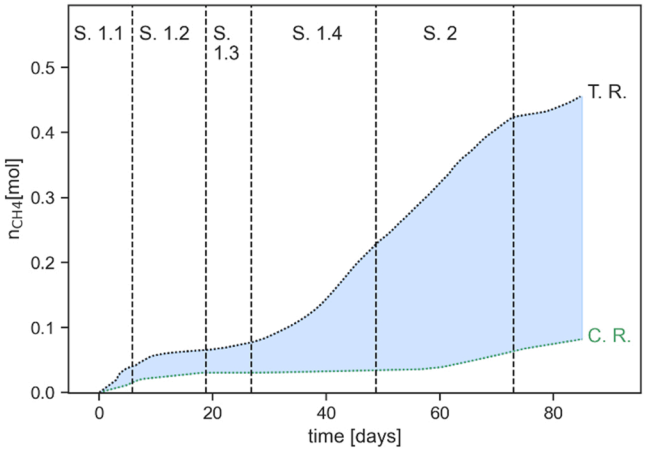

Fig. 4. Cumulative methane production in the Test and Control Reactor over the stages of the experiment outlined in Table 1; the blue area highlights the surplus of methane produced in the Test Reactor compared to the Control Reactor.

The substrate COD concentration (Fig. 3d) dropped during the adaptation stage (days 0–49) by an overall 24.4% in the Test Reactor and by 35.6% in the Control Reactor, indicating only a partial inhibition related to the presence of syngas on the digestion of organic compounds in the liquid phase. This is further supported by a reduction in volatile solids of 29.7% in the Test Reactor and of 33.5% in the Control Reactor (Table 2). Both observations indicate that already during adaptation under continuous syngas feeding, the cultures could perform a considerable organic load reduction, thus pointing to the possibility of conducting adaptation processes in concomitance with waste treatment in scaled-up continuous processes.

The maximum methane production in the Test Reactor during the adaptation stage was achieved on day 44 and was equal to 0.5 $mmol{\cdot}h^{-1}$ (195 $mL{\cdot}L^{-1}_{Liquid}{\cdot}d^{-1}$). At this point, the fundamental role of syngas feeding in increasing the reactor methane productivity is evident from the significantly higher methane production rate in the Test Reactor compared to the Control reactor as shown in Fig. 3a and from Fig. 4 by the visible difference in the increment of the cumulative methane production between the two reactors. Between days 42 and 45, a conversion of H_2 and CO molar flow of 88% and 80% could be observed, respectively, and an electron balance performed on the gas phase showed that on day 44 the electron-equivalent methane flow was 6% in excess of the converted CO and H_2, clearly indicating additional methane production from the liquid substrate, most likely from the depletion of acetic acid as suggested by the declining trend observable in Fig. 3c.

Between days 44 and 49, then, methane production fluctuated in the Test Reactor between 0.39 and 0.5 $mmol{\cdot}h^{-1}$, corresponding to methane productivity of the adapted culture between 150 and 195 $mL_{CH4}{\cdot}L^{-1}_{Liquid}{\cdot}d^{-1}$. During this period, the conversion efficiencies of H_2 and CO stayed respectively in the ranges 88 ± 3% and 80 ± 5% while the average volumetric nitrogen-free outlet gas composition obtained was 14 ± 2% H_2, 31 ± 6% CH_4, 11 ± 1% CO and 44 ± 6% CO_2. Furthermore, during the last 5 days of the adaptation stage (days 44–49), the flow of methane in terms of electron-equivalents stayed in a range between + 6% and − 15% of the total converted CO and H_2 moles indicating a relatively stable methanogenic performance of the adapted culture with high selectivity towards methane. The stable methane productivity at this stage can be also visualized by the linear increase of the cumulative methane production in the Test Reactor (Fig. 4). These results demonstrated that already after 44 days of continuous adaptation phase, syngas could be converted at high efficiencies in the presence of the BSY substrate, suggesting that relatively fast continuous adaptation processes could be developed for the industrial scale by making use of a waste substrate with zero cost.

3.2.2. Continuous co-digestion

On day 49, the syngas and substrate metabolism of the culture in the

Test Reactor were regarded as sufficiently stable to allow moving on to the final stage of the experiment, where the liquid substrate was also fed in semi-continuous mode. The Test and Control Reactor were operated in this mode from day 49 to day 69. Between days 49 and 56, the methane production rate in the Control Reactor remained almost constant between 0.014 and 0.02 $mmol \cdot h^{-1}$ and showed an increase only from day 56 to day 59, reaching 0.05 $mmol \cdot h^{-1}$, and subsequently growing up to 0.08 $mmol \cdot h^{-1}$ on day 69 (Fig. 3a). Thus, after a lag phase of about 10 days, likely related to substrate hydrolysis kinetics, the conversion of BSY allowed the volumetric methane productivity of the Control Reactor to increase from 3% up to 16% relative to the methane production in the Test Reactor on day 44 (i.e., the maximum methane production recorded in the final stage of continuous adaptation). On the other hand, the methane production in the Test Reactor remained relatively constant after the beginning of the liquid substitutions (Fig. 4a), witnessing the role of syngas as the primary electron moles and carbon source in this stage. In specific, the methane production rate fluctuated around 0.4 mmol/h between days 49 and 60. In the same period, the H_2 and CO conversion in the Test Reactor remained almost constant in the range of $88 \pm 10\%$ and $80 \pm 10\%$, respectively, demonstrating relatively high conversion efficiency and satisfactory process stability over the shift between continuous feeding of sole syngas (continuous adaptation) and continuous co-feeding of syngas and BSY. Process stability at this stage can be also visualized by the linear increase in the cumulative methane production in the Test Reactor (Fig. 4). Between days 60 and 63, methane production in the Test Reactor peaked at 0.6 $mmol \cdot h^{-1}$ (volumetric productivity: 233 $mL_{CH4} \cdot L_{Liquid}^{-1} \cdot d^{-1}$) to progressively decline down to 0.3 $mmol \cdot h^{-1}$ on day 69. During the peak in methane production (day 62), the methane output represented 128% of the converted gas-phase electron moles, indicating that a considerable fraction of methane stably originated from the liquid phase. The 10–12 days-long delay in methane formation with respect to the start of continuous BSY feeding was thus likely related to the initial hydrolysis kinetics of BSY, which was then followed by a quick enhancement in methanation activity. Furthermore, around the period of highest methane production (i.e., days 58–65), the acetic acid concentration (Fig. 3c) increased and subsequently stabilized around a value of 15 $mmol \cdot L_{Liquid}^{-1}$ excluding the hypothesis of an increased methane production deriving from the depletion of accumulated acids. After day 69, then, the methanogenic activity showed a drastic drop accompanied by a simultaneous drop in H_2 and CO uptake. Such observation suggested that a nutrient limitation was likely taking place. The BSY injections were thus interrupted to verify the hypothesis of a limitation in nutrients in the absence of further substrate addition. As sulfur is an essential element for the growth and metabolism of methanogens involved in syngas biomethanation processes [8,25] and based on previous experience of process disruption in case of sulfur limitation [8], it was expected that the culture could be recovered by supplementing pure Na_2S. Shortly after nutrient addition (25 $mg \cdot L_{Liquid}^{-1}$ Na_2S on day 77), the methane production and syngas uptake were restored respectively at 0.25 $mmol_{CH4} \cdot h^{-1}$ and above 80%, thus validating the hypothesis of sulfur limitation and indicating that the control of secondary nutrients is essential to ensure the stability and efficiency of a continuous co-digestion process.

Overall, before the methane production drop caused by nutrient limitation (i.e., between day 49 and 69) the Test Reactor yielded an average methane production rate of 0.4 $mmol \cdot h^{-1}$ and a maximum methane production rate of 0.6 $mmol \cdot h^{-1}$, corresponding respectively to 150 and 233 $mL_{CH4} \cdot L_{Liquid}^{-1} \cdot d^{-1}$. Under the continuous syngas inflow applied (1.1 $L \cdot L_{Liquid}^{-1} \cdot d^{-1}$ with 45 v% H_2 and 20 v% CO), and the organic loading rate of 0.55 $g_{VS} \cdot L_{Liquid}^{-1} \cdot d^{-1}$ (corresponding to 1.18 $g_{COD} \cdot L_{Liquid}^{-1} \cdot d^{-1}$) the ratio between the volume of CH_4 produced and the volume of consumed CO and H_2 was on average 0.2 and hit a maximum of 0.3. As a comparison, Yang et al. [13] fed a 4 L CSTR with 1.3 $L \cdot L_r^{-1} \cdot d^{-1}$ syngas along with 3.5 $g_{VS} \cdot L_r^{-1} \cdot d^{-1}$ of food waste yielding 1.485 $L_{CH4} \cdot L_r^{-1} \cdot d^{-1}$ at mesophilic conditions. They registered a methane volume ratio on consumed H_2 and CO of 1.14, i.e., 3.8 times the maximum volumetric ratio registered in the present study (0.3) likely due to a higher organic loading and COD conversion rate. On the other hand, Zupančič et al. [17] investigated the digestion of sole BSY-rich brewery wastewater as substrate in an upflow anaerobic reactor. Having an influent COD of 9.8 $g \cdot L^{-1}$ (16.5 $g \cdot L^{-1}$ in the present study) and an organic loading rate of 12.6 $g_{COD} \cdot L_r^{-1} \cdot d^{-1}$ (i.e., over 10 times higher compared to the present study) they obtained a methane productivity of 2.62 $L_{CH4} \cdot L_r^{-1} \cdot d^{-1}$, which is about 15 times higher compared to the present study. Thus, these comparisons show that there is substantial space for optimization of the methane yield obtained by co-digestion of BSY and syngas in the present study, which could be achieved by increasing the organic loading rate under concentrations of added trace elements that allow for a stable metabolism. However, the concentration limits of BSY in the influent should be carefully evaluated to avoid other forms of inhibitions coming from solids overloading and ammonia formation as outlined by Zupančič et al. and Sosa-Hernández et al. [16,17]. Furthermore, the drastic loss of methane production witnessed, and the strong nutrient limitation subsequently verified highlight the need for further detailed studies into the management of secondary nutrients concentration. Such studies should specifically focus on possible additional waste streams that can supplement the necessary nutrients into a BSY-syngas co-digestion process while averting the use of high-cost synthetic sources. Nevertheless, the present assessment of a process comprising a syngas-exposed adaptation phase followed by continuous co-digestion proves the effectiveness of biomethane production via simultaneous BSY treatment and syngas biomethanation, where further optimizations of methane productivities could set the premises for scaled-up integrated processes. Importantly, the observation of a stable transition from an adaptation stage without continuous liquid substitution to a continuous feeding stage may be especially relevant to larger-scale processes subject to seasonal patterns of waste generation, where cost-effective microbial adaptation procedures can be initiated shortly in advance of the expected waste stream production regimes without the need to implement ad-hoc standalone adaptation processes.

3.2.3. Analysis of microbial community composition

The microbial community analysis was carried out on samples collected from both reactors on day 0 (inoculation), day 49 (end of adaptation stage by continuous syngas feeding in the Test Reactor), and day 67 (after 1.3 HRT of BSY feeding) to assess the changes caused by the applied operating conditions and provide further evidence on the co-digestion of syngas and BSY.

The dominant phyla in all collected samples were *firmicutes* and *bacteroidota* whereas in the Control Reactor at day 67 also *thermotogota* occupied a significant share, indicating a substantial presence of hydrolytic species [26] in both reactors and during all the stages of operation. However, the processes in both reactors at all stages were being carried out by a quite large array of species as the Shannon diversity index at the genus level (normalized to the maximum possible diversity in the database of identified genera) remains in the range 0.60–0.65 for all samples. Still, despite being characterized by comparable within-sample diversity, the composition of the microbial communities was affected by the operating conditions applied to the reactors as shown by Principal Component Analysis (PCA) at the genus level. Indeed, as visualized by the distance between vectors in Fig. 5b, the initial culture (D0) underwent a change after the first 49 days of operation during which the culture was exposed to continuous syngas feeding in the Test Reactor and to nitrogen in the Control Reactor. The sole exposure to gas in the Test Reactor did not generate substantial differences in the microbial communities between the Test and Control Reactor on day 49 likely because there was no washout of inactive cells. On the other hand, the co-feeding of syngas and BSY in the Test Reactor between days 49 and 67 led the microbial composition in the Test Reactor to diverge significantly from the one in the Control Reactor, where only BSY was fed. The most present genus in the Test and Control

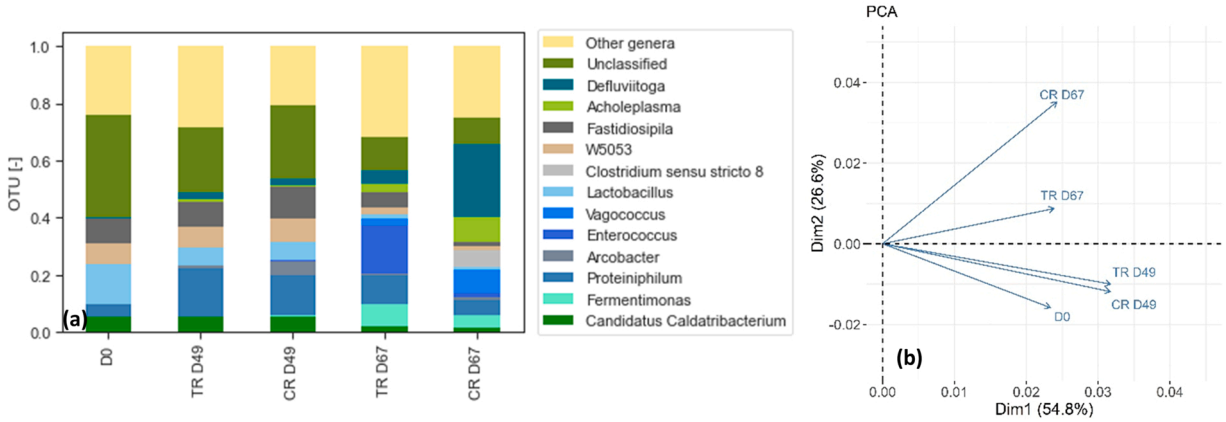

Fig. 5. (a) Microbial community composition in terms of abundance of OTU at genus level at day 0 (D0-inoculation), day 49 in the Test and Control Reactor (respectively TR D49 and CR D49- end of adaptation stage), day 67 in the Test and Control Reactor (respectively TR D67 and CR D67-after 1.3 HRT of BSY feeding). (b) Visualization of PCA analysis of the OTU abundance matrix at genus level: vectors representing the microbial community composition of the analyzed samples are projected in the space defined by the principal components characterized by the highest variance.

reactors on day 49 was *Proteiniphilum* (abundance 17% and 14%, respectively) which was significantly enriched compared to the composition of the initial culture (5% abundance). Other known genera appearing with a high abundance in the Test and Control reactors on day 49 were: *Fastidiosipila*, *Lactobacillus*, *Candiditatus Caldatribacterium* (Fig. 5a). These bacteria also shared a high abundance of operational taxonomic units (OTU) in the initial culture, whereas the number of reads mapping to *Fastidiosipila* increased by 1.3 times in the Control Reactor within the first 49 days and remained constant in the Test Reactor. The abundance of OTU of *Lactobacillus* was almost halved in both reactors on day 49 compared to the initial culture, whereas the abundance of *Candidatus Caldatribacterium* remained almost constant from day 0 (Fig. 5a). The enrichment of *Proteiniphilum* is a clear indication of BSY metabolism as it was previously detected in an anaerobic digestion reactor treating brewery wastewater [27]. Furthermore, bacteria belonging to both *Proteiniphilum* and *Fastidiosipila* have been reported to perform proteolytic and acetogenic activity [27]. The presence of this type of activity in both reactors seems then to confirm the observed acetic acid production (Fig. 3c), also considering the favorable conditions provided by the high protein content of BSY [18]. On the other hand, *Lactobacillus* and *Candidatus Caldatribacterium* are known for their ability to degrade sugars to acetate and ethanol [28,29]. Regarding the archaeal population, the number of reads mapping to the domain of archaea was 1.9% in the Test Reactor and 1.4% in the Control Reactor on day 49, with the dominant genus being *Methanosarcina.* The abundance of OTU related to the genus *Methanosarcina* in the Test Reactor on day 49 was 1.3 times higher than in the Control Reactor. The larger abundance is likely related to the presence of syngas, as species belonging to the genus *Methanosarcina* have previously been reported as hydrogenothrophic methanogens but also aceticlastic and carboxydotrophic methanogens [13,30]. Furthermore, on day 49 the culture in the Test Reactor showed the presence of the genus *Desulfotomaculum* (4% abundance), which typically presents rather versatile species showing sulfate-reducing, hydrogenotrophic, and carboxydotrophic acetogenic abilities, as well as carboxydotrophic hydrogenogenic abilities [30]. This suggests that sulfur metabolism played an important role during the biological conversion of syngas at this stage. Sulfur might have originated from the digestate used as inoculum and/or by the addition of sulfuric acid for pH control between days 0 and 49.

The continuous feeding of BSY in the Test and Control reactor led to a significant difference in the composition of the microbial community both between the two reactors at the end of the continuous feeding process (day 67), and between the compositions registered on day 67 and 49, as shown by the relatively large distance between the corresponding vectors on Fig. 5b and by the visible differences in compositions displayed in Fig. 5a. On day 67 in the Test Reactor the microbial community still showed a high abundance of OTUs related to *Proteiniphilum* and *Fastidiosipila*. However, the dominant genus of the community in the Test Reactor on day 67 was *Enterococcus*, a genus to which several sugar-metabolizing species belong [31]. The genus *Fermentimonas*, which is composed of hydrolytic acetogenic species [32] was found with a significant abundance of OTUs in the Test Reactor on day 67. On day 67 in the Test Reactor, 0.8% of the reads at the domain level were mapped to methanogenic *archaea*. Among these, the main genera were with similar abundance *Methanosarcina* and *Methanoculleus*, revealing the possibility of simultaneous aceticlastic, carboxidotrophic, and hydrogenotrophic methanogenic activity in the Test Reactor [26, 30]. At this stage, the genus *Desulfotomaculum* was still present but with lower abundance compared to day 49 (1.2% of the identified genus-level OTU abundance) hinting to lower sulfur concentration in the liquid phase after 1.3 HRT of co-feeding of BSY and syngas. On the other hand, the genus *Alkalibaculum,* which is characterized by the ability of acetogenic metabolism of glucose, yeast extract, and CO [33], was enriched during the co-feeding stage reaching up to 3.8% abundance of OTU. These observations indicate an adaptation to the new operating conditions of the syngas metabolizing microbial community during the co-feeding stage, which, however, still required sulfur as a key nutrient for survival as witnessed by the drop in syngas consumption and methane production rate observed after day 67 (see Figs. 3a and 3b). On the contrary, in the Control Reactor on day 67, the dominant genus was *Defluviitoga*, which is composed of mainly fermentative species capable to degrade complex molecules like cellulose, chitin, and xylan [6,34]. Other genera present in significant quantities were *Proteiniphilum*, *Fermentimonas*, and *Acholeplasm*a, which was also reported as a common strain in anaerobic digestors [35]. Additionally, 0.3% of reads mapped to methanogenic archaea on day 67 in the Control Reactor, i.e., less than a half with respect to the Test Reactor on day 67 witnessing the important role of syngas as substrate for methane production also at this stage of operation.

In summary, the analysis of the microbial community composition demonstrated the co-existence of microorganisms able to metabolize simultaneously BSY and syngas and highlighted substantial differences in microbial composition between the first stage, in which only syngas was fed continuously, and the second stage, with syngas and BSY continuous co-feeding. Furthermore, the microbial composition analysis showed that syngas co-digestion with BSY was carried out by a rather diversified community in which the whole metabolic process was carried out via hydrogenotrophic, carboxydotrophic, fermentative, and aceticlastic pathways. Future work should investigate the microbial community composition of syngas co-digestion with BSY under

optimized substrate loading rates.

4. Conclusions

In the present study, we investigated the potential of an integrated syngas biomethanation and brewery spent yeast (BSY) co-digestion process to deliver cost-effective biomethane production in a lab-scale CSTR system that was operated for 85 days overall. Culture adaptation to the simultaneous digestion of syngas and liquid substrate was carried out following multiple strategies, i.e., in batch bottles and directly in a CSTR. All adapted cultures showed the ability to consume simultaneously syngas and the COD content of the liquid substrate at relatively high conversion efficiencies thus demonstrating that BSY is a suitable substrate to conduct cost-effective mixed microbial cultures adaptation processes under syngas exposure. The culture adapted in the CSTR system was then operated with continuous syngas and BSY feeding, reaching the highest methane productivity recorded in this study (233 $mL_{CH4} \cdot L_{Liquid}^{-1} \cdot d^{-1}$) and thus pointing to the high potential of BSY for the use in continuous co-digestion processes. A microbial community composition analysis also showed that methanogenic activity related to the conversion of syngas and BSY to methane was carried out via simultaneous hydrogenotrophic, carboxydotrophic, fermentative, and aceticlastic activity by a relatively diversified microbial community. Future work should focus on the enhancement of methane productivity and process stability by making use of trace elements derived from additional waste streams that can fulfill the operating cost constraints of possible industrial scale-ups.

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CRediT authorship contribution statement

Pietro Postacchini: Conceptualization, Methodology, Data curation, Investigation, Visualization, Writing – original draft, Writing – review & editing. **Lorenzo Menin:** Supervision, Conceptualization, Visualization, Methodology, Writing – original draft, Writing – review & editing. **Stefano Piazzi:** Methodology, Writing – review & editing. **Antonio Grimalt-Alemany:** Supervision, Conceptualization, Writing – review & editing. **Francesco Patuzzi:** Supervision, Writing – review & editing, Funding acquisition. **Marco Baratieri** Supervision, Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Data Availability

Data will be made available on request.

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Supplementary information

Raw DNA sequences can be found on the NCBI database under the BioProject access number PRJNA924235.

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